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EFFECTS OF WOUND BACTERIA ON POSTBURN ENERGY METABOLISM

ANNUAL SUMMARY REPORT

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SUMMARY

The increase in resting energy expenditure of a rat following a 30% total body surface burn is related to the process of wound colonization. Seeding the wound with a non-virulent strain of *P. aeruginosa* (NVP) results in a very reproducible, largely homogenous, localized, gram-negative wound infection. Seeded rats express a 15-40% increase in metabolism during the first postburn week, while burned unseeded rats experience little change in metabolic rate over this same period. The goal of this study was to identify the afferent signals initiated by wound bacteria and responsible for postburn hypermetabolism. Last year, we demonstrated that endotoxin was neither a principal afferent or the primary inducer of other mediators of this response. This year, we found that heat-killed NVP and *S. epidermidis* (SE) continuously infused under the unseeded burn wound (10^6 - 10^8 /hr) failed to recreate the hypermetabolic response to either NVP or SE wound seeding. There is a general relationship between core temperature and metabolic heat production following injury. The NVP-seeded rat is febrile on postburn days (PBDs) 1 and 2, but the hypermetabolism on PBDs 3-4 and 7-8 occurs at normal central body temperature. Efforts to alter postburn hypermetabolism by improving humoral and cell-mediated immunity were largely unsuccessful. The inflammatory response of the NVP-seeded wound was reduced by topical and systemic hydrocortisone treatments. This increased the incidence of invasive infection, but failed to alter the hypermetabolic response of the non-bacteremic rats. Using a sensitive, two-step bioassay (LBRM), interleukin-1 (IL-1) activity was uncovered in the serum of burned rats. There was no relationship between IL-1 levels and the metabolic response to injury. This IL-1 activity apparently developed during the clotting process, since it could not be detected in plasma. A non-cytotoxic, low molecular weight (less than 10 kD), relatively heat stable, inhibitor of IL-1 (INH) was identified in the serum of burned rats. The INH was effective against rat IL-1 and human monocyte-derived IL-1, but its suppressive effect could be offset by increasing IL-1 concentration. Taken together, the data indicate that afferent mediators of postburn hypermetabolism are initiated in response to active bacterial growth in the wound. These signals are non-thermoregulatory in nature and resistant to changes in the animal's immunocompetence. There does not appear to be enough "free" IL-1 in the circulation to act as an afferent mediator of postburn hypermetabolism. The biologic role of the INH is undefined, but suggests that systemic IL-1 effects are determined by the balance achieved between this cytokine and its circulating inhibitors.

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<p>The rise in resting metabolic rate (RMR) of the 30% total body surface burned rat is related to degree of localized wound infection. Seeding the wound with non-virulent <i>P. aeruginosa</i> (NVP) at the time of injury resulted in a 20-40% increase in RMR during the first postburn week. Rats with unseeded wounds expressed little or no change in RMR by postburn days (PBDs) 7-8. The degree of hypermetabolism varied with the number of live microorganisms in the wound, but could not be reproduced by continuous infusion of dead bacteria or endotoxin into the subcutaneous tissue beneath the unseeded wound. Burned rats were febrile on PBDs 1-2, but usually not thereafter. Efforts to alter postburn hypermetabolism by improving humoral or cell-mediated immunity were unsuccessful. The reduction of wound inflammation with topical and systemic hydrocortisone increased invasive infection without affecting RMR of non-bacteremic rats. Interleukin-1 (IL-1) was uncovered in the serum of burned rats, but there was no relationship between IL-1 level</p>			
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and RMR. A low molecular weight inhibitor of rat and human IL-1 (INH) was also identified in the serum of burned rats. The data indicate that afferent mediators of postburn hypermetabolism originate in the wound in response to localized bacterial colonization. These signals are non-thermoregulatory and resistant to variations in immunocompetence. IL-1 does not appear to be an important circulating mediator of postburn hypermetabolism, but its biological significance is masked by the presence of naturally occurring inhibitors.

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life Sciences, National Research Council (NIH Publication No. 86-23, Revised 1985).

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INTRODUCTION

The rise in energy expenditure following trauma varies with the extent of injury and reaches its greatest magnitude in the burn patient. A young, otherwise healthy patient with a 50% total body surface burn (TBSB) can have a resting metabolic rate twice normal with every major system in the body working at an accelerated rate.

Catecholamines are the principal efferent mediators of postburn hypermetabolism (1), but the afferent signals which initiate and sustain the increased sympathoadrenal activity are largely undefined. The basic relationship between wound size and the degree of hypermetabolism suggests that these signals originate in the wound, and there is good evidence that they travel via the circulation to affect central nervous system control of energy expenditure (2,3).

Historically, burn patients were thought to be hypermetabolic because they were cold (4-6). The increase in metabolic heat production was believed to compensate for the increased evaporative heat loss from the wound. Heat balance following thermal injury is further complicated by the fact that patients are febrile and have to produce more heat to raise body temperature above normal. While the data collected on small burns supported this thesis, gradually more and more investigators began to question the significance of a thermoregulatory basis for postburn hypermetabolism. The problem with this concept developed when postburn hypermetabolism could not be eliminated by either blocking wound evaporative heat loss or raising ambient temperature into the patient's thermoneutral zone (7-10). Since the metabolic rate at thermoneutrality is minimal and independent of changes in ambient temperature (11), the extra metabolism at thermoneutrality must be considered the energy cost of injury.

In the past, investigators have separated the metabolic responses to burn injury into those occurring before and after bacteremia or sepsis. They do so in order to distinguish between the effects of injury and the superimposed infection. While this is an important clinical distinction, it leads to the concept that the non-bacteremic patient is "free of infection" (12) and fails to address any systemic metabolic effects of bacteria prior to their actual entrance into the blood stream. Since the wound is not sterile during the hypermetabolic phase of injury and infection alone produces metabolic and neuroendocrine adjustments similar to those in thermal injury (13), wound bacteria and/or their products may be important metabolic stimuli in the "uninfected" patient. This is the hypothesis upon which this two-year study was founded.

Bacterial contamination of the burn wound increases the metabolic rate of rats. This was first suspected when seeding 30% TBSB wounds with non-virulent bacteria raised the metabolic rate of non-bacteremic animals above that of unseeded, burned controls (14). In this same study, topical antimicrobial treatments of seeded wounds reduced the hypermetabolic response of non-bacteremic rats. Subsequent work revealed that, when burn wounds were allowed to colonize in a natural, spontaneous manner, there was a significant correlation between wound bacterial count and the increase in resting oxygen consumption of burned, non-bacteremic rats (15). There was little change in metabolic rate of the burned rat until wound bacterial concentration reached 10^4 /gram, suggesting that animals with sterile wounds would not be very hypermetabolic. Variations in the data, however, revealed that factors other than the number of viable bacteria affect this relationship between the process of wound colonization and postburn hypermetabolism.

Since bacteria appear to initiate the metabolic response while still confined to the burn wound, they must release, or cause the release of other humoral mediators. These afferent signals may be bacterial products (enzymes, toxins, etc.) or cytokines produced by host inflammatory cells in response to bacteria or their products. Endotoxin is a prime candidate in either case, since this lipopolysaccharide (LPS) component of the cell wall of gram-negative bacteria increases metabolic rate upon entering the circulation (16) and is also a potent inducer of cytokine production (17). Interleukin-1 (IL-1) and cachectin/tumor necrosis factor (TNF) are two cytokines believed responsible for many systemic responses to injury and infection. Our focus has been on the metabolic effects of LPS and IL-1 released in the colonized burn wound.

Recent studies revealed that endotoxin released in the colonized burn wound does not serve as a circulating mediator or the principal inducer of other mediators of postburn hypermetabolism in the rat (18). This was demonstrated in three ways. First, rats with localized gram-positive wound infections are just as hypermetabolic as those with gram-negative infections. Second, continuous subcutaneous infusion of LPS beneath the unseeded burn wound (at rates which were 10 times the estimated maximum rate of LPS release in the infected burn wound) failed to raise resting metabolic rate above that of the burned controls. Third, the hypermetabolic burned animals were not endotoxemic.

The establishment of a relationship between wound bacterial count and postburn hypermetabolism and the elimination of wound endotoxin as a principal afferent mediator were the primary accomplishments of the first year of this contract. In the second year, the principal goals were to (a) investigate the effects of dead bacteria on postburn hypermetabolism; (b) evaluate the relationship between metabolic heat production and body temperature; (c) assess the effects of changes in

immunocompetence on the metabolic response to wound colonization; and (d) determine whether there are measurable levels of interleukin-1 (IL-1) in the blood of the non-bacteremic burned rat.

MATERIALS AND METHODS

Study Design. The basic protocol involved measuring resting metabolic rate and colonic temperatures of rats before and for one to two weeks after full-thickness 30% TBSB. Variations in wound bacterial density were accomplished by seeding some wounds with non-virulent microorganisms while allowing others to colonize spontaneously. Topical antimicrobials were not used. Blood and spleen cultures were performed on all animals. Since the study was designed to identify the effects of localized wound infection, bacteremic or septic animals were not included in the data analysis.

Animals. The animals selected for study were 4-7 month old, male Sprague-Dawley rats (Hilltop, Scottdale, PA) weighing 400-600 grams. They were housed in individual cages and had access to food and water throughout. A 12-hour light/dark cycle was maintained. Ambient temperature ranged from 25-28°C while animals were not under study.

Burn Injury and Wound Seeding. The rat was anesthetized (sodium pentobarbital, 4.5 mg/100 g body weight, i.p.) and its pelage clipped from the back and flanks. It was then placed in a mold exposing 30% of the total body surface, and a full-thickness burn produced over this area by immersion in 98°C water for nine seconds. Burn wounds were either left unseeded or seeded with a non-virulent *P. aeruginosa* (NVP) (19) at the time of injury. All 18-hour seeding cultures contained 10^8 bacteria/ml of tryptic soy broth, and one ml of the broth was spread over the entire wound.

Metabolic and Temperature Measurements. Resting metabolic rate was determined by indirect calorimetry using respiratory gas exchange measurements collected over a 3-hour interval. The animals were placed in individual, flow-through respiration chambers located in a temperature controlled cabinet. (A complete description of the apparatus and measurement routine is provided in the previous annual report.) These measurements were performed at thermoneutrality for the rat - 30°C before injury and 32°C after injury. They began 2-3 weeks before injury and continued until reproducible normal resting values were obtained. After injury, studies were performed on postburn days (PBD's) 3-4, 7-8, and 14-15 and the data averaged for each two-day block. Metabolic rate was expressed in Watts/m² of body surface area.

One hour after the metabolic study, colonic temperatures of burned and unburned rats were measured six centimeters from the

external anal sphincter as previously described (15). This permitted a direct comparison between burned and unburned animals at the same time of day and in the same thermal environment. Peritoneal temperature was monitored through the use of a small indwelling radiotransmitter (Model VM-FH, Mini-Mitter, Co., Sunriver, OR). Output frequency of these transmitters varied as a function of temperature, while variations in amplitude provided an index of animal activity. This biotelemetry system permitted continuous recording of core temperature and activity of unrestrained animals while they were resting quietly in the metabolic chamber or active overnight in the animal quarters. Transmitter frequency and amplitude were monitored at 5-minute intervals over the period of observation (a 3-hour metabolic study or a 12-hour overnight run) and the data analyzed on a personal computer utilizing Dataquest II software. Temperature and activity data were reported as an average of each measurement period.

The transmitters were implanted under sterile conditions, and no measurements were performed until at least two weeks after surgery. All transmitters were calibrated in a stirred water bath before implantation and the end of each complete study and considered accurate to $\pm 0.1^{\circ}\text{C}$.

Bacteriology. Quantitative blood and spleen cultures were performed on all animals as previously described (15). Basically, aortic blood was drawn aseptically and 4-5 drops placed on a plate of tryptic soy agar with 5% sheep's blood (Difco). The spleen was removed, dipped in alcohol, and flamed to remove surface bacteria. It was then placed in a sterile tissue grinder containing 5 ml of sterile water. The number of viable bacteria in 0.1 ml of the homogenate was determined by serial dilution and back plating on sheep's blood agar. All plates were read after 24 and 48 hours incubation at 37°C . Only rats with negative blood and spleen cultures were included in the data analysis.

Subcutaneous Infusion of Dead Bacteria. Eighteen-hour cultures of NVP or S. epidermidis (SE, ATCC 12228) were pelleted by centrifugation and resuspended in 10 ml of sterile saline. After two such washes, a small sample was obtained for quantitative culture and the remainder heated at 56°C for 10 minutes. After cooling, another sample was taken for culture, and the cells repelleted, and stored at -4°C for 24 hours. The 24-hour cultures verified that all bacteria had been killed and provided an estimate of the number killed. Based on this information, the cells were resuspended in sterile saline and the suspension placed in small osmotic pumps (Alzet Model 2ML, Alza, Palo Alto, CA). The pumps were inserted into the subcutaneous tissue of the midscapular region (under an unseeded burn wound) immediately after injury. Each pump provided a continuous infusion of approximately 10^7 - 10^8 heat-killed NVP or SE cells/hr for the next two weeks. (This underestimated the actual infusion rate, because it was based only on the number of bacteria killed. It was chosen, however, because this is about the rate at which

bacteria die in the infected burn wound.)

Hydrocortisone Treatments. Systemic glucocorticoid administration reduces the accumulation of inflammatory cells in the wound and the rate of IL-1 production by these cells (20-22). The problem is that glucocorticoids are also calorogenic. Therefore, the dose administered must be large enough to reduce white cell infiltration and IL-1 production in the wound but less than that required to increase total body metabolism. Three different hydrocortisone treatments were utilized to achieve this delicate balance. A thin coat of 0.5% hydrocortisone cream (Hydrocortisone Cream, NMC Laboratories) was applied daily to the seeded burn wound. A 0.5% hydrocortisone aerosol (Caldecort, Pennwalt Corp.) was sprayed on the wound once daily. Subcutaneous injections of hydrocortisone (Hydrocortone, Merck, Sharp & Dohme) were given three days before the burn, on the day of burn and on PBD 3. The total dose was 0.6 mg/g body weight.

Active Immunization Against NVP. Three protocols were used to immunize rats against live NVP microorganisms. The first, originally described by Walker et al. (23), involved weekly i.p. injections (X4) of an 18-hour culture (0.02 ml, 0.05ml, 0.1 ml and 0.2 ml). When these rats became septic before injury, a second set of animals were immunized against NVP using the same injection schedule but a reduced number of bacteria/injection (1/1000 dilution of the 18-hour culture). While this proved to be a successful immunization procedure, the third protocol was established in an attempt to increase antibodies titers. In this protocol, live NVP bacteria were mixed with Freund's complete adjuvant (10^6 NVP/ml) and 0.2 ml injected i.p. Two more injections were given at monthly intervals, but Freund's incomplete adjuvant was used. Controls for the weekly injection protocol were injected with sterile saline. Controls for the monthly protocol were not injected.

Serum Antibody Measurements. An enzyme-linked immunosorbent assay was performed on serum samples from immunized and unimmunized rats. The 96-well microtiter plates were prepared by first adding 50 ul of poly-L-lysine solution (PLL, 1 mg/100 ml of phosphate buffered saline (PBS), pH 7.2). After 30 minutes at room temperature, the PLL solution was discarded and 50 ul of a live, pre-washed NVP bacterial solution (10^8 /ml PBS) was added to each well. The plate was centrifuged at 2000 rpm for 20 minutes at 4°C and 50 ul of a 0.5% glutaraldehyde solution (in cold PBS) was added to each well and left at room temperature for 15 minutes. The plate was washed twice with PBS and the wells filled with 100 mM glycine in a 0.1% bovine serum albumin (BSA) solution and left at room temperature for 30 minutes. The plate was washed twice in PBS and stored in 0.1% BSA frozen at -20°C. The actual assay began by adding 200 ul of 10mM dibasic sodium phosphate with 0.9% NaCl and 1% BSA to each well and incubating this mixture for 45 minutes at room temperature. The plate was then washed (X5) with PBS and 100 ul of rat antiserum, diluted in PBS with 1% BSA, was added to each well and incubated at room

temperature for two hours. After another five washes with PBS, 200 μ l of peroxidase assay mixture (10 mg o-phenylenediamine, 4 μ l 30% H_2O_2 , 10 ml 0.1 M citrate buffer, pH 4.5) was added to each well. The antibody titer is the highest dilution yielding a yellow-colored product.

Cyclophosphamide and Indomethacin Treatments. Low dose cyclophosphamide and indomethacin restore cell mediated immunity (CMI) in burned mice (24). To determine whether the immunosuppression accompanying burn injury affects resting metabolic rate, burned, NVP-seeded rats received cyclophosphamide (2.4 mg/kg/day) or indomethacin (0.5 mg/kg/day). Controlled release of both drugs was achieved by subcutaneous implantation of specially designed pellets (Innovative Research of America, Toledo, OH) at the time of injury. These pellets released the drugs at a constant rate for three weeks. Control animals received a placebo. The level of CMI was assessed by measuring the sensitivity to 2,4-dinitrofluorobenzene (DNFB) as described by Hansbrough et al. (24) Burned rats were sensitized on PBD 8 by applying 2 ml of 0.5% DNFB (in 4:1 acetone:olive oil) to the shave abdomen. Six days later, ear thickness was measured to nearest 10^{-4} inch with vernier calipers and 0.2 ml of 0.2% DNFB applied to each ear. CMI was assessed 24 hours later by measuring the degree of ear swelling.

Interleukin-1 Assay. Serum was fractionated over Sephadex G 50-80 column and that portion containing molecules in the 10-30 kD range was pooled and simultaneously concentrated and dialyzed (Micro-Protein Dialysis-Concentrator, Biomolecular Dynamics, Beaverton, OR). The nominal molecular weight cutoff for the dialysis membrane was 10 kD.

Serum IL-1 activity was assessed using a sensitive, two-step bioassay (25). This assay detects IL-1 by its capacity to convert an interleukin-2 (IL-2) nonproducer murine cell line (LBRM-33-1A5, ATCC) to an IL-2 producer. The amount of IL-2 produced is determined by the proliferative response of IL-2 dependent murine cytotoxic T-lymphocytes (CTLL, ATCC). In the first step, 100 μ l of 5×10^5 LBRM cells/ml of complete media, 50 μ l serum and 50 μ l of phytohemagglutinin (2.5 μ g/ml PHA, Sigma, St. Louis, MO) are placed in each well of a 96-well microtiter plate (Costar, Cambridge, MA) and incubated at 37°C in a 5% CO_2 , 100% humidity environment for 24 hours. In the second step, 100 μ l of complete media containing 2×10^5 CTLL cells/ml and 100 μ l of the LBRM supernatant are added to each well of the 96-well plate and incubated for 24 hours as described above. At 20 hours, 0.5 μ Ci 3H -thymidine (Amersham, Arlington Heights, IL) was added. Four hours later, the cells are harvested and thymidine uptake measured in a liquid scintillation counter (Beckman LS 9000, Beckman, Irvine, CA). The amount of IL-1 activity in each serum sample was expressed as a multiple of the background CTLL-2 proliferation. This Stimulation Index (S.I.) was calculated by dividing thymidine uptake (cpm) of the CTLL-2 cells stimulated with serum and PHA by that produced by PHA stimulation alone.

Interleukin-1 Inhibitor Assay. IL-1 studies suggested that a low molecular weight inhibitor of IL-1 (INH) was present in the dialysate. The INH was recovered from the dialysate by lyophilization, re-suspension in distilled water, fractionation over a Sephadex G-10 column to remove salts, re-lyophilization and resuspension in RPMI 1640 medium without L-glutamine or fetal calf serum. The suppressive effects of the INH were tested in the LBRM assay by adding back increasing quantities of INH to rat serum containing a fixed amount of IL-1 activity. A fixed quantity of INH was also tested against increasing doses of human, monocyte cell line derived IL-1. Heat stability of the INH was assessed before and after heating at 56°C for 10 minutes. LBRM and CTLL cell viability was tested by trypan blue exclusion.

Data Analysis. Multiple unpaired t-tests with the Bonferroni adjustment (26) were used to assess differences between groups of burned animals at each postburn interval. Paired t-tests with the Bonferroni adjustment were used to identify postburn changes within the same group of animals. All values are expressed as mean \pm standard error of the mean (SEM). A p value less than 0.05 was considered statistically significant.

RESULTS

Changes in Core Temperature. Core, or deep body temperature, was monitored in two ways - (a) a single measurement from the colon one hour after the metabolic study and (b) continuous measurement from the peritoneum while the rat was resting quietly in the afternoon (from 1300 to 1615 hours) and active overnight (from 1800 to 0600 hours). Colonic temperatures are provided later with the corresponding metabolic data of each experiment. In general, colonic temperature of the non-bacteremic, NVP-seeded rat hovered in the high normal range from PBD 1 to PBD 3. Peritoneal temperatures were frequently elevated at the beginning of the afternoon metabolic study, but fell into the preburn range over the three-hour period of observation. Figures 1 illustrate the typical effects of burn plus NVP-seeding on peritoneal temperatures of two rats. Overnight temperatures exceeded afternoon values by roughly 1 °C before and after injury. This was associated with higher levels activity during the overnight study. Burned, NVP-seeded rats were consistently febrile over the first one or two days after injury. This increase in core temperature was evident in the afternoon and overnight and generally associated with a decrease in activity. The fever rapidly abated in most rats but appears to be returning in Rat B by PBD 3. Overnight activity levels quickly returned to normal following injury. Afternoon activity levels tended to be slightly higher when the rats were studied in the cooler (24 °C) animal quarters than in the warmer (32 °C) metabolic chamber.

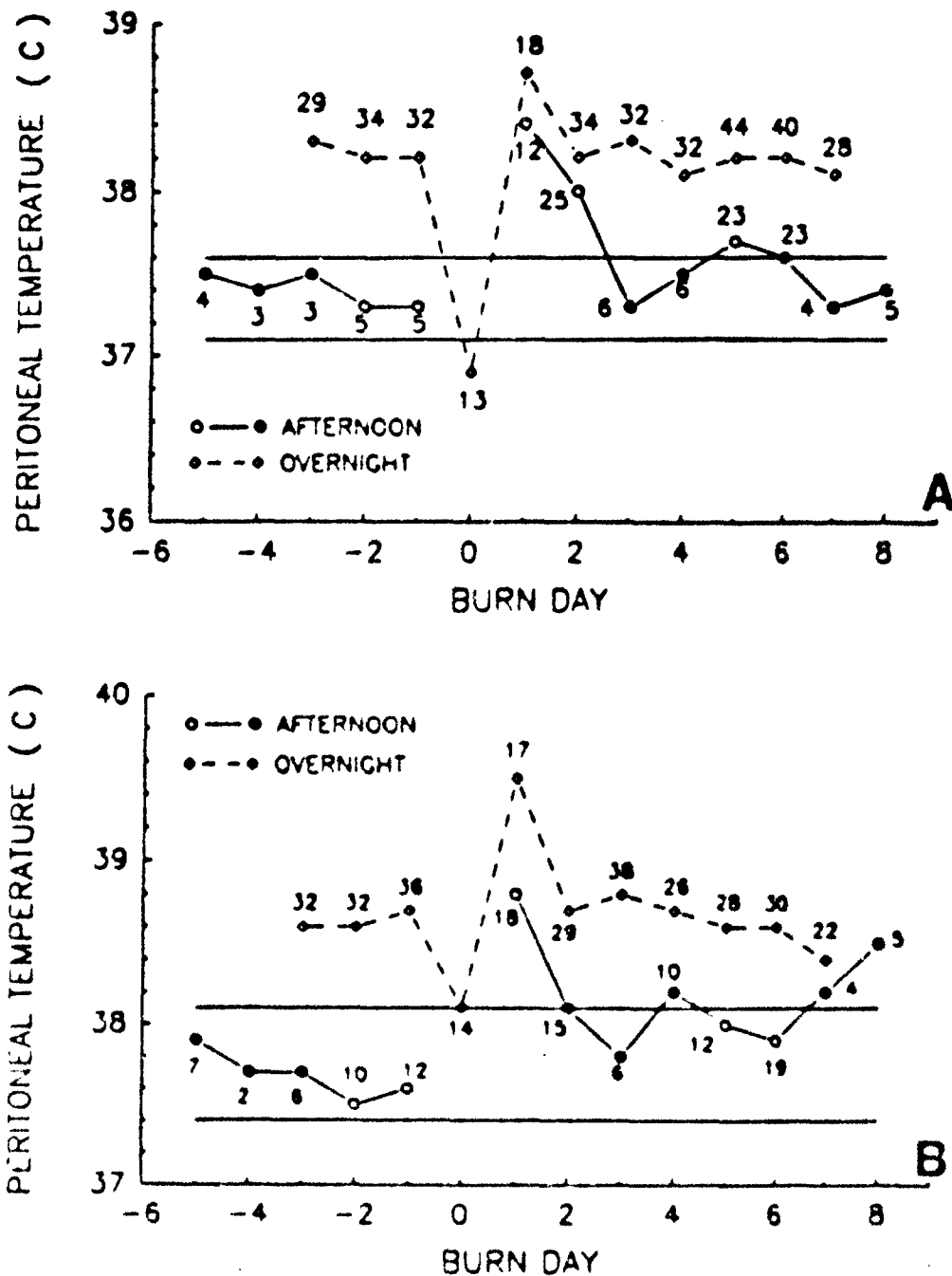


Figure 1. NVP seeding of burn wounds resulted in a transient febrile response. Average peritoneal temperature and activity were determined in the afternoon (1300-1615 hrs) in the metabolic chamber (●) or animal quarters (○). Overnight studies were conducted in the animal quarters. Ambient temperature averaged 27-28 °C in the animal quarters, 20 °C in the chamber before burn and 32 °C after injury. Average activity scores (arbitrary units) are listed at each data point. Two rats are shown.

Effects of Dead Bacteria. Resting metabolic rate was measured in 52 rats before and after a 30% TBSB. Seventeen animals received a continuous subcutaneous infusion of heat-killed SE bacteria (10^7 - 10^8 /hr) under the unseeded burn wound, 18 rats were infused in a similar manner with heat-killed NVP bacteria, and 17 burned controls were infused with sterile saline. The infusion of dead bacteria increased metabolic rate above that of the burned controls, but there was no difference in the metabolic response to NVP and SE bacteria (Table I). Colonic temperature was not different among the three burned groups nor significantly elevated above unburned rats.

TABLE I. Effects of subcutaneous infusion of heat-killed bacteria on metabolic rate of burned, unseeded rats.

Group	Number	Resting Metabolic Rate (W/m ² , mean±SEM)		
		PBD 0*	PBDs 3-4	PBDs 7-8
1. Controls	17	32.6±0.5	33.3±0.5	34.7±0.4
2. Dead NVP	18	32.0±0.8	37.9±0.7	37.6±0.6
3. Dead SE	17	31.6±0.5	37.7±0.8	37.9±0.8
P value < ‡		NS	.05	.05

* Calculated from the last three studies prior to injury.

‡ Burn versus controls, unpaired t-tests with Bonferroni

Effects of Hydrocortisone Treatments. Twenty-four burned, NVP-seeded rats were treated with topical or systemic hydrocortisone. Wound histology revealed a qualitative reduction in inflammatory cell density. Thirteen of these rats were bacteremic on PBD 8. This is significantly higher than the 10% infection rate normally encountered following a 30% total body surface, NVP-seeded burn. The effects of glucocorticoid administration on resting metabolic rate, colonic temperature and body weight of the 11 treated, non-bacteremic animals are compared to those of 13 burned, untreated, non-bacteremic controls in Table II. There was no significant difference in the metabolic responses between treated and untreated burned rats, but the treated animals lost more weight over the period of observation (15 g/day) than the untreated animals (8 g/day).

Effects of Immunization. Three different protocols were used to immunized rats against NVP bacteria. The first caused 15 unburned animals to become septic, and was not repeated. In the second, nine animals received weekly i.p. injections (X4) of live bacteria, while nine rats were injected with sterile saline. In the third protocol, 15 rats received monthly i.p. injections of live bacteria. The bacteria were mixed with Freund's complete adjuvant for the initial injection, and Freund's incomplete adjuvant for the last two injections. Fifteen control animals were not injected. All burn wounds were seeded with NVP at the

time of injury. There was no significant difference in resting metabolic rate, colonic temperature or body weight between immunized and unimmunized rats following injury (Table III). There was no correlation between postburn hypermetabolism and antibody titer on PBD 8. Antibody titers ranged from 1/100 to 1/1000 preburn (n = 6), 1/300 to 1/1000 in the unimmunized rats on PBD 8 (n = 14) and 1/300 to 1/10,000 in the immunized rats on PBD 8 (n = 24). Freund's adjuvant increased postburn antibody titers (1/300 - 1/1000 without Freund's and 1/1000 - 1/10,000 with Freund's).

TABLE II. Effects of topical and systemic hydrocortisone treatments on metabolic rate, colonic temperature and body weight of burned, NVP-seeded rats.

Metabol/g Rate (W/m ²)	PBD 0 [†]	PBD 3-4	PBD 7-8
Topicals [*]	32.4±0.9	35.6±0.8	37.4±1.1
Systemic ^{**}	32.8±1.0	39.0±2.5	39.0±1.2
Untreated ^{***}	32.6±0.7	35.9±0.9	37.8±1.0
P value < ^④	NS	NS	NS
Colonic Temperature (°C)			
Topicals	36.8±0.1 ^{④⑤}	37.1±0.2	37.0±0.1
Systemic	36.8±0.1	37.0±0.2	37.0±0.3
Untreated	36.8±0.1	37.2±0.1	37.2±0.1
P value <	NS	NS	NS
Body Weight (grams)			
Topicals	526±14	441±14	405±11
Systemic	492±15	430±18	386±18
Untreated	507±12	454±9	440±12
P value <	NS	.05	.05

^{*} Calculated from last three studies prior to injury

^{*} 0.5% hydrocortisone cream (n = 3) or aerosol spray (n = 5) applied daily to the burn wound

^{**} 3 subcutaneous injections of hydrocortisone (3 days before burn, day of burn, and PBD 3, n = 3)

^{***} Topical controls were left undisturbed (n = 10), systemic controls received injections of sterile saline (n = 3)

^④ Treated versus untreated, unpaired t-tests plus Bonferroni

^{④⑤} PBD 0 temperatures are from 36 unburned rats measured at the same time and in the same environment as the burned animals

TABLE III. Effects of active immunization against NVP bacteria on metabolic rate, colonic temperature, and body weight of burned, NVP-seeded rats.

Metabolic Rate (W/m ²)	PBD 0 [#]	PBD 3-4	PBD 7-8
Immunized	32.4±0.3	37.9±0.6	37.0±0.6
Unimmunized	31.9±0.4	39.8±1.1	38.5±0.8
P value <*	NS	NS	NS
Colonic Temperature (°C)			
Immunized	36.7±0.1**	37.1±0.1	36.8±0.1
Unimmunized	36.9±0.1	37.4±0.1	37.3±0.1
P value <	NS	NS	NS
Body Weight (grams)			
Immunized	536±8	479±8	477±7
Unimmunized	543±8	476±8	476±8
P value <	NS	NS	NS

* Calculated from last three studies prior to burn

* Multiple unpaired t-tests with Bonferroni

** PBD 0 temperatures from unburned controls measured at the same time and in the same environment as the burned animals

Effects of Cyclophosphamide and Indomethacin. Twenty-one burned, NVP-seeded rats received cyclophosphamide, nine received indomethacin and 21 controls were not given either drug. The metabolic response to burn wound colonization was not altered by either drug over the two week period of observation (Table 4). Likewise, treated and untreated animals lost weight at the same rate (8 g/day) following injury. DNFB sensitization caused a 55% increase in ear thickness of unburned rats compared to a 0-30% in the burned animals. There was no difference in the DNFB response between treated and untreated burn groups.

TABLE IV. Effects of cyclophosphamide and indomethacin on metabolic rate and body weight following thermal injury.

Metabolic Rate (W/m ²)	PBD 0	PBD 3-4	PBD 7-8	PBD 14
Cyclophosphamide*	30.8±0.4	38.1±0.7	39.2±0.5	45.2±1.1
Indomethacin**	32.6±0.7	41.6±1.1	41.4±1.8	44.8±2.5
Placebo	30.6±0.3	36.6±0.6	38.3±0.5	45.3±0.0
P value <*	NS	NS	NS	NS
Body Weight (grams)				
Cyclophosphamide	536±8	470±8	449±8	427±10
Indomethacin	550±12	482±15	472±15	441±14
Placebo	518±9	456±9	441±8	407±9
P value <	NS	NS	NS	NS

* 2.4 mg/kg/day

** 0.5 mg/kg/day

* Treatment versus placebo, unpaired t-tests with Bonferroni

Serum Interleukin-1. Fractionated serum samples from four burned rats processed no IL-1 activity. When the fractionated burn serum was pooled, concentrated and dialyzed, IL-1 activity appeared in pool B (15 to 30 kD) and pool C (below 15 kD) but not in pool A (above 30 kD). Six separate assays on pool B serum from nine unburned rats and 24 burned rats (13 NVP-seeded and 11 unseeded) revealed a significant increase in serum IL-1 activity following burn injury (Figure 2).

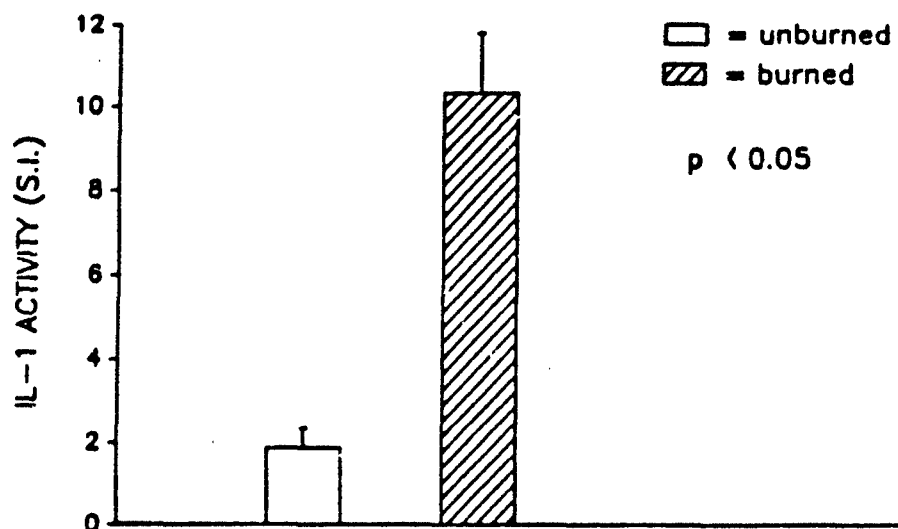


Figure 2. IL-1 activity in serum samples of burned and unburned rats. Fractionated and concentrated/dialyzed samples were tested in the LBRM assay. IL-1 activity was measured by the mitogenic response of lymphocytes (^3H -thymidine uptake in cpm) and expressed as the stimulation index (S.I.) where $\text{S.I.} = (\text{cells} + \text{PHA} + \text{serum}) - (\text{cells} + \text{PHA}) / (\text{cells} + \text{PHA})$.

There was no difference in the IL-1 activity between seeded and unseeded rats. Adding serum samples to CTLL-2 cells did not cause them to proliferate. Likewise, adding NVP endotoxin or recombinant TNF-alpha to the LBRM incubation media failed to induce IL-2 production. IL-1 activity was reduced by heating serum samples (70 °C for 30 minutes, $n = 3$).

Serum Interleukin-1 Inhibitor. When the IL-1 dialysis material was concentrated and desalted as previously described, it depressed rat serum IL-1 activity in a dose dependent manner (Figure 3). The INH had no effect on the proliferation of IL-2 stimulated CTLL-2 cells (Figure 4). It was not cytotoxic for either the LBRM or CTLL cell lines. Finally, the suppressive effects of the INH was inversely related to amount of IL-1 present in the sample (Figure 5). In this case, the inhibition was overcome by adding increasing amounts of human, monocyte derived IL-1 beta.

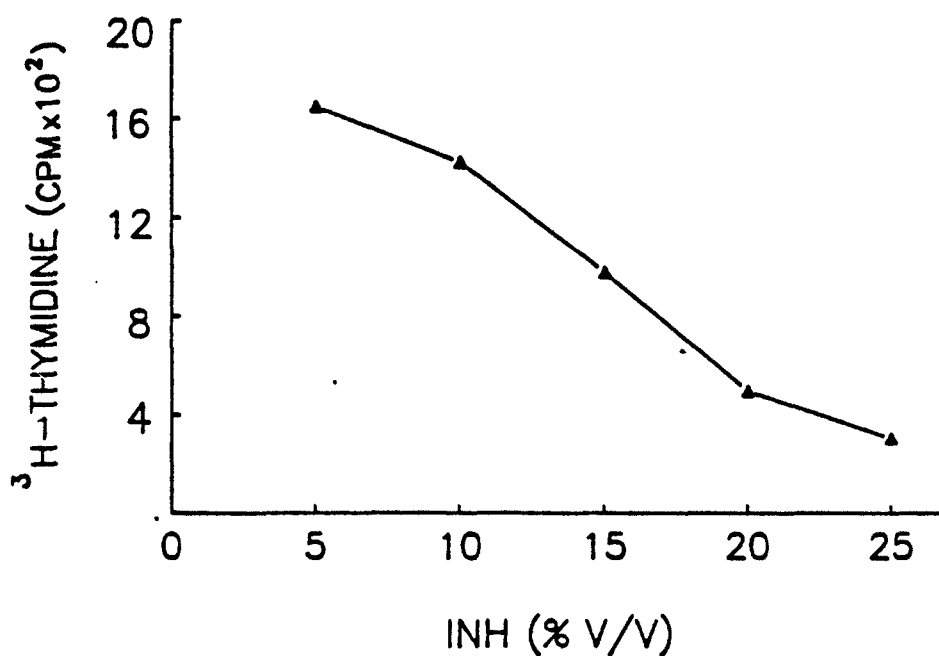


Figure 3. Effects of the low molecular weight inhibitor of IL-1 (INH) on rat serum IL-1 activity. IL-1 activity was measured in the LBRM assay by proliferation of murine lymphocytes (³H - thymidine uptake in cpm). The INH dose is expressed as a % of culture volume (INH %v/v).

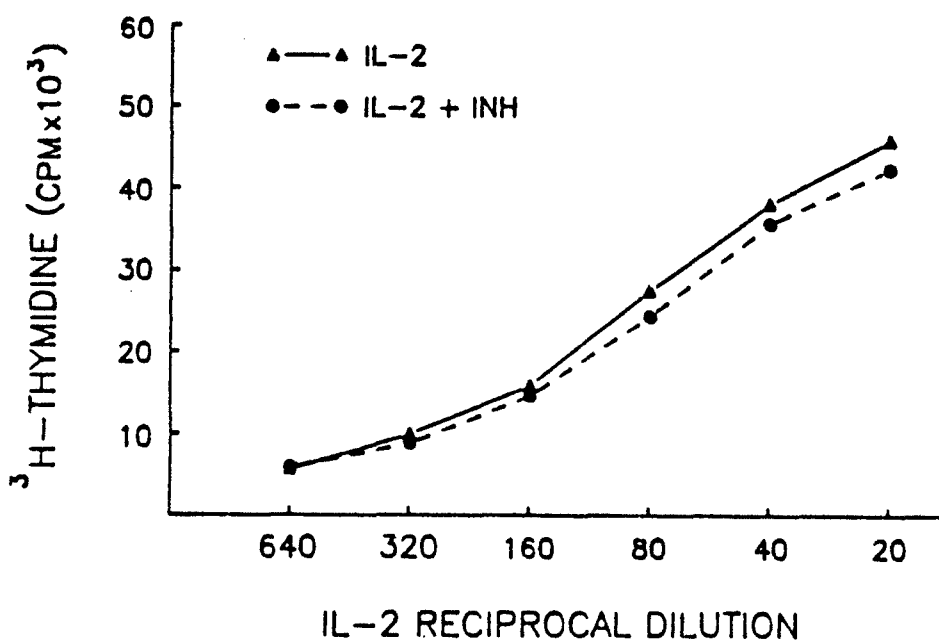


Figure 4. The low molecular weight inhibitor (INH) has no effect on the proliferation of IL-2 dependent CTLL cells (³H-thymidine uptake in cpm). IL-2 dose-dependent proliferation was measured both with (●--●) and without (▲—▲) a fixed amount of the INH.

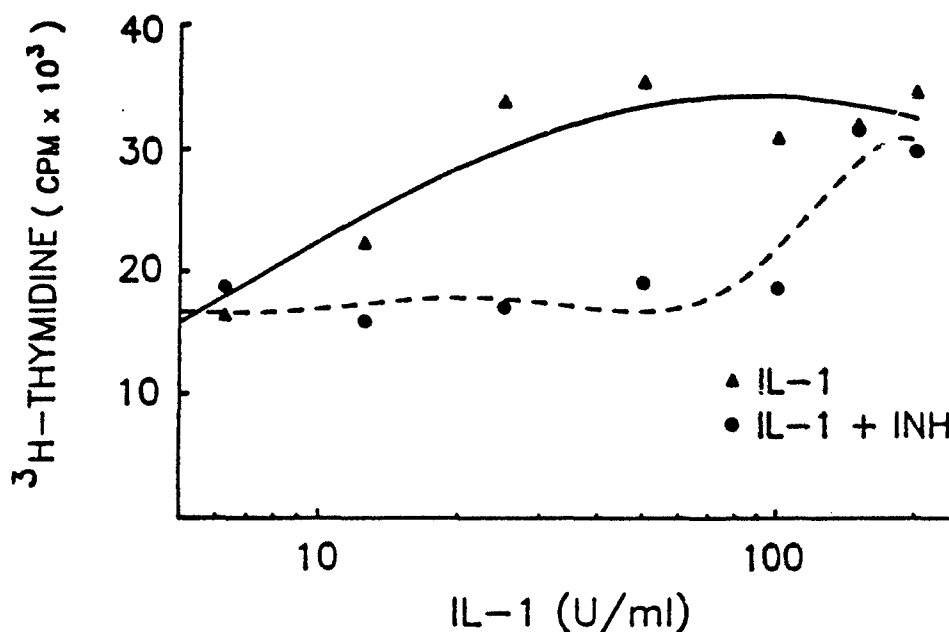


Figure 5. The effect of the low molecular weight inhibitor (INH) on human IL-1 activity. Human monocyte-derived IL-1beta activity was measured in the LBRM assay by the proliferation of murine lymphocytes (^3H - thymidine uptake in cpm) with (●) and without (▲) a fixed amount of rat INH.

DISCUSSION

In the first contract year, we demonstrated that the hypermetabolic response to burn injury varied with the degree of localized wound colonization (15). This relationship was highly variable, however, indicating that factors other than the number of live bacteria in the wound affect resting energy expenditure. We also discovered that endotoxin released in the colonized wound was neither an obligatory afferent mediator nor the major inducer of other mediators of postburn hypermetabolism in the non-bacteremic rat (18). Humans are exquisitely more sensitive to endotoxin than rats, however, so the clinical significance of wound endotoxin cannot be derived from rat data alone. It is possible that such differences in endotoxin sensitivity explain the greater hypermetabolic response of humans with the same size burn. Even if this is the case, the rat data suggest that a sizable portion of the hypermetabolic response to wound colonization can be unrelated to local release of endotoxin.

This year we continued the search for afferent mediators of postburn hypermetabolism in the 30% TBSB rat model. The principal hypothesis was that these signals originate in the burn wound as the result of localized bacterial growth. This is supported by the model which is not hypermetabolic during the first postburn week unless the wound is seeded with bacteria

(14,15,18). Seeding wounds with either the non-virulent form of P. aeruginosa or S. epidermidis provides an excellent means to study the effects of localized wound infection, since these animals express a 15-40% increase in resting energy metabolism without becoming bacteremic during the first week.

There is a general relationship between the increase in metabolic rate and the increase in core temperature of the non-bacteremic burned rat (15). For this reason, any search for afferent mediators of postburn hypermetabolism must begin with an evaluation of the thermoregulatory status of the model. If seeding the burn wound causes the rat to become febrile, then the increase in metabolic rate may be simply a thermoregulatory response designed to raise body temperature in the face of accelerated heat loss through the wound.

When large numbers of animals are studied, the average colonic temperature of seeded rats is statistically above those of unburned or burned unseeded animals (15). This difference is usually less than 0.5 °C and rarely evident when smaller groups are studied (Tables II and III). Through the use of biotelemetry, we were able to monitor core temperature of unrestrained rats over extended periods of time. We observed a transient increase in peritoneal temperature of the non-bacteremic, NVP-seeded rat which developed during PBD 1 and largely disappeared by PBD 3 (Figure 1). On postburn days 3-4 and 7-8, peritoneal temperature was frequently above normal when the animals entered the metabolic chamber, but it usually fell into the normal range over the three-hour period of measurement. With its large surface-to-mass ratio, the deep body temperature of the rat is far more volatile than it is in larger animals and humans. For this reason, the elevated peritoneal temperature at the beginning of the metabolic study most likely reflects a response to handling superimposed on the basic hypermetabolic background.

Since, postburn hypermetabolism was commonly demonstrated at normal body temperature, this increase in heat production cannot be considered part of a fever response. While both thermal and non-thermal metabolic drives exist and contribute to the hypermetabolic state of the burned patient, the non-thermal drive appears to be a more consistent (and possibly the more sensitive) response to localized burn wound infection. This separation and the relative significance of thermal and non-thermal metabolic drives have been described earlier in both patients and animals (8,10).

After eliminating thermal afferents, we focused on other potential humoral mediators. The first choice was endotoxin, but it proved to play a relatively insignificant role in the hypermetabolic response of the rat (18). One explanation is that endotoxin is only one of many bacterial factors which serve as afferent mediators or inducers of mediators. To administer only this one component may reduce the strength of the overall stimulus to a point that it is no longer effective.

We attempted to increase stimulus strength by continuously infusing heat-killed NVP (or SE) bacteria into the subcutaneous tissue beneath the unseeded burn wound. This increased the metabolic rate above that of unseeded controls (Table I), but not substantially above that generated by endotoxin infusion alone (18). These results suggest that living bacteria and/or their products are more important hypermetabolic stimuli in the wound. Liu (27), for example, has shown that *P. aeruginosa* produce several extracellular toxins significantly more potent than endotoxin. Since SE-seeded rats are as hypermetabolic as the NVP-seeded animals, the proposed "active" components of the hypermetabolic stimulus do not appear to be specific to a particular bacterial strain. It may be that products released in the process of killing are also necessary ingredients of an afferent signal.

A second potential mediator of postburn hypermetabolism is interleukin-1. This small polypeptide is produced by activated phagocytic cells during inflammation, infection and antigenic challenge (17). It acts both as a local immunomodulator and as a "wound hormone". Its systemic effects include fever, acute phase protein production, shifts in plasma trace metals, anorexia, increased ACTH release, slow wave sleep, leukocytosis and many other features of localized infection. The stable, non-bacteremic burn patient manifest most, if not all, of the systemic responses of IL-1, but there is little evidence of this cytokine in the blood of burn patients. Kupper et al. (28) identified a 15-20 kD form of IL-1 in blister fluid of burned patients, while Clowes and collaborators (29,30) found a 4.2 kD IL-1 fragment in the plasma of three burn patients. Others, using monoclonal antibodies, have been unsuccessful in their efforts to find IL-1 in patient blood (personal communication, Dr. A.D. Mason, Jr., USAISR, Fort Sam Houston, TX).

IL-1 activity was uncovered in the serum of burned rats, and the associated findings suggest that IL-1 was responsible for the IL-1 activity observed. First, the IL-1 activity was recovered from serum fractions containing molecules in the same molecular weight range as IL-1. Second, IL-1 and the serum factor responsible for the IL-1 activity are both heat labile. Third, the IL-1 activity could not be explained by other factors (endotoxin, TNF or IL-2) which may mimic IL-1 in the LBRM assay.

The IL-1 activity was evident only after the serum had been fractionated, and these fractions pooled, concentrated and dialyzed. Such processing presumably uncovered the IL-1 activity by removing naturally produced inhibitors and/or concentrating the active compound. Inhibitors of IL-1 dependent thymocyte proliferation have been previously identified in serum (31), urine (32-35) and cell culture supernatants (36-40). Most of these inhibitors have molecular weights above 30 kD, and would have been removed by the fractionation process alone. The inability to detect IL-1 activity in the fractionated serum, however, suggests either that circulating levels of IL-1 were

below the threshold for the LBRM assay or that a low molecular weight inhibitor was removed by the dialysis-concentration procedure.

The recovery of IL-1 activity involved an eight fold increase in serum concentration. Diluting the sample back up to its original volume reduced IL-1 activity but did not eliminate it. This indicates that only part of the inability to detect serum IL-1 activity before concentration resulted from low circulating levels of the cytokine. It further suggests that a low molecular weight inhibitor was removed by the dialysis step. This was subsequently confirmed when the addition of concentrated, desalted samples of the dialysate reduced IL-1 activity in rat serum in a dose dependent fashion (Figure 3). Since it was present in the dialysate, the inhibitor (INH) had to have a molecular weight below the 10 kD cutoff of the dialysis membrane. It proved to be relatively heat stable, non-cytotoxic and specific for IL-1 (Figure 4). The capacity to overcome the suppressive effects of the INH by the addition of IL-1 suggests some form of competitive inhibition (Figure 5). This may occur (a) by binding to IL-1 to block its active site, (b) by interfering with IL-1 receptor binding at the target cell or (c) by disrupting some post binding function of IL-1 in the target cell. All such mechanisms have been previously described for other IL-1 inhibitors.

An IL-1 inhibitor of similar size has been identified in the blood of patients with acquired immunodeficiency syndrome (AIDS) (37). Likewise, a small molecular weight inhibitor has been reported in the serum of burn patients, but no details were provided (41). The clinical implications of the INH are unknown, but could potentially include many immunologic and physiologic adjustments to injury. In some incidences it could be protective and in others quite destructive. Taken together, the IL-1 and INH data indicate that the systemic effects of IL-1 are not determined by its rate of production or the amount that enters the circulation but rather by the balance achieved between IL-1 and its inhibitors.

The question remaining is whether, on balance, IL-1 is an afferent mediator of postburn hypermetabolism. There are now several lines of evidence that suggest it is not. First, the serum IL-1 levels uncovered in this study were unrelated to metabolic rate (42). Second, the infusion of IL-1 into unburned rats has had mixed effects on metabolic rate (43,44). Similarly, the injection of an IL-1 inducer in normal humans yielded indirect evidence for systemic IL-1 release without causing any change in energy expenditure (45). Finally, there does not appear to be much circulating IL-1 in the hypermetabolic patient or rat. Cannon et al. (46) have recently revealed that serum IL-1 concentration exceed plasma levels and demonstrated that IL-1 was produced in vitro during the clotting process. Such in vitro IL-1 production must have contributed to the IL-1 activity observed in burn serum samples, because we subsequently have been unable to find any IL-1 activity in the plasma of burned animals.

Once again, however, there is very preliminary evidence of plasma INH activity (unpublished data). So, one should not conclude there is no IL-1 in plasma simply because there is no measurable IL-1 activity. Likewise, IL-1 may bind to the INH or other plasma proteins thereby altering its biologic activity and its recognition by monoclonal antibodies. Consequently, all one can safely say is that there does not appear to be much "free" IL-1 in the circulation.

We were able to reduce inflammation (and presumably IL-1 production) in the NVP-seeded wound with both topical and systemic hydrocortisone treatments, but the result was an increased incidence of invasive infection (13/24 treated animals were bacteremic on PBD 8). The hypermetabolic response of the remaining non-bacteremic animals was comparable to that of the untreated controls, indicating that the dose administered was not calorogenic (Table II). The accelerated weight loss which accompanied hydrocortisone treatment may be explained by either the generalized catabolic (wasting) or anorexic effects of glucocorticoids. The important point is that it did occur without an associated energy cost. It also demonstrated that some of the topically applied hormone entered the circulation to exert systemic effects. Consequently, even had there been a reduction in metabolic rate following topical treatment, we would have been unable to argue that it occurred because of a localized reduction in IL-1 production. While it may be possible to localize the effects by further reductions in the topical glucocorticoid dose, time did not permit this kind of exploration.

The metabolic response to burn wound colonization was also unaffected by three separate attempts to alter the immune capacity of burned NVP-seeded rats. We were able to produce a low grade immune response to NVP bacteria, but the immunized rat was just as hypermetabolic as the unimmunized control. It is possible that a metabolic effect may develop at more substantial levels of immunization, but this will require a more effective immunization protocol than currently employed.

Attempts to improve cell-mediated immunity (CMI) by low dose administration of cyclophosphamide and indomethacin were equally ineffective. In our hands, DNFB sensitization resulted in about a 55% increase in ear thickness of the uninjured rat. This same test failed to cause consistent ear swelling in either the treated or untreated burned groups. From this, we can only conclude that the CMI of the NVP-seeded rat was not altered by either cyclophosphamide or indomethacin. Once again, variations in administered dose or injection schedules may be required.

There turned out to be another problem with this protocol. Metabolic rate of both treated and untreated burned animals climbed markedly following the application of DNFB. This was not the result of DNFB, because it developed in rats receiving only the acetone:olive oil vehicle. So, even if we had been able to affect an improvement in CMI with either treatment, it would be

difficult to demonstrate metabolic consequences using this assay for CMI.

The inability to modify postburn hypermetabolism by a variety of immunoregulatory techniques was frustrating, but it clearly demonstrated both the complexity of the afferent signal and the resiliency of the response. Between the rapidly expanding in vitro data and clinical data is an ever increasing void where fewer and fewer researchers tread. Consequently, we now can clone molecules faster than we can identify either their biologic or clinical relevance. The USAMRDC is to be complimented for its unique support of this very difficult yet extremely important exploration of the middle ground.

CONCLUSIONS

The connection between the process of wound colonization and postburn hypermetabolism of the rat is now firmly established, but the afferent mediators remain undefined. While the model is a localized, gram-negative burn wound infection, there is strong evidence that endotoxin or other components of the bacterial cell wall are not principal stimuli. Rather the data suggest that living microorganisms and their products are required for full development of the afferent signal. The increase in energy expenditure is not to maintain the febrile state, for it persists long after the acute febrile response has abated. The IL-1 present in the serum of burned rats appears to be a product of in vitro clot formation. The discovery of a low molecular weight IL-1 inhibitor offers yet another mechanism by which to control the systemic biologic and immunologic effects of this wound hormone. The inability to modify postburn hypermetabolism by a variety of immunoregulatory techniques demonstrates both the complexity of the afferent signal and the resiliency of the response.

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